



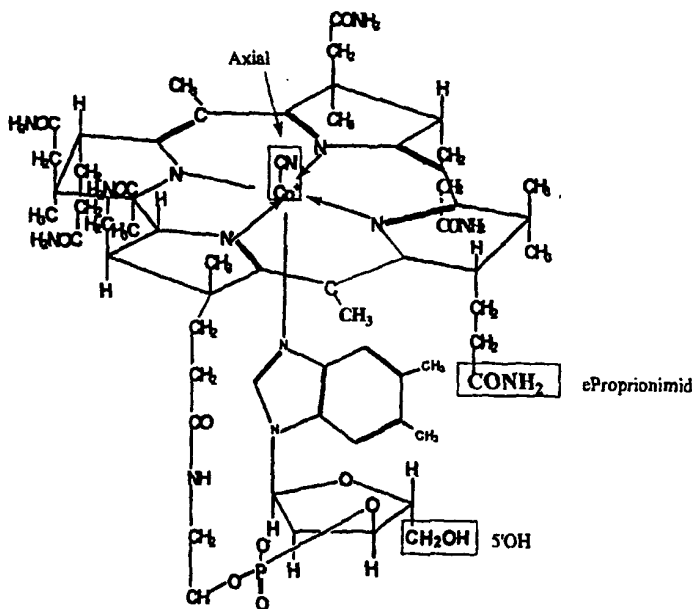
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(21) International Application Number: PCT/AU99/00462 (22) International Filing Date: 11 June 1999 (11.06.99) (30) Priority Data: PP 4050 12 June 1998 (12.06.98) AU (71) Applicant (for all designated States except US): BIOTECH AUSTRALIA PTY. LIMITED [AU/AU]; 28 Barcoo Street, Roseville, NSW 2069 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): RUSSELL-JONES, Greg [AU/AU]; 23 Greenfield Avenue, Middle Cove, NSW 2068 (AU). MCEWAN, John [AU/AU]; 241A West Street, Blakehurst, NSW 2221 (AU). (74) Agents: STEARNE, Peter, Andrew et al.; Davies Collison Cave, Level 10, 10 Barrack Street, Sydney, NSW 2000 (AU).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.	

(54) Title: VITAMIN B₁₂ DERIVATIVES AND METHODS FOR THEIR PREPARATION

(57) Abstract

This invention relates to methods for preparing vitamin B₁₂ (VB₁₂) derivatives suitable for linking to a polymer, nanoparticle or therapeutic agent, protein or peptide. The methods involve reacting the 5'OH group of VB₁₂ or an analogue thereof with an active carbonyl electrophile and subsequently obtaining said VB₁₂ derivatives. The invention also relates to novel VB₁₂ derivatives, VB₁₂ derivatives prepared by the methods of the present invention and uses thereof in the preparation of polymer complexes or nanoparticles.

Conjugation sites of VB₁₂

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VITAMIN B₁₂ DERIVATIVES AND METHODS FOR THEIR PREPARATION

5

TECHNICAL FIELD

The present invention generally relates to novel derivatives of vitamin B₁₂ carrier molecules
10 for the delivery of therapeutic substances by administration of a complex comprising these
substances linked to vitamin B₁₂ (VB₁₂) or an analogue thereof. The invention also generally
relates to novel methods for preparing VB₁₂ derivatives. More particularly, the invention
relates to reactions of the 5'OH group of VB₁₂ with electrophiles to prepare these VB₁₂
derivatives.

15

BACKGROUND OF THE INVENTION

An oral delivery mechanism for peptides is described in International application
PCT/AU86/0299 (WO87/02251) based on recent work undertaken by one of the current
20 inventors. The mechanism utilises at least one carrier molecule to which an active substance
is linked to transport the active substance from the intestinal lumen into the circulatory
system. VB₁₂ and analogues thereof function as ideal carrier molecules by using the natural
VB₁₂ uptake system, mediated by the binding of VB₁₂ to intrinsic factor (IF), to transport the
active substance/VB₁₂ complex. Once delivered into the lymphatic drainage system or
25 serum, the complex substantially retains the bioactivity of the native active substance.

More recently conjugates of VB₁₂ with drugs, cytotoxins and MRI agents, have been used in
the detection and treatment of tumour cells. For normal cellular uptake of vitamin B₁₂
(cobalamin, Cbl, VB₁₂), the vitamin must first bind to the plasma protein transcobalamin II
30 (TCII). Following binding of Cbl to TCII the resultant TCII-Cbl complex binds with high
affinity to receptors on the surface of cells and is internalized by the cell via a process called
receptor-mediated endocytosis (RME). Once inside the cell the Cbl is enzymatically
modified to form two coenzymes, which are in turn used for two essential metabolic
pathways. One pathway involves the methylation of homocysteine in the *de novo* synthesis

of methionine, and is catalyzed by methionine synthase. The other pathway involves the rearrangement of methylmalonyl CoA to succinyl CoA, and is catalyzed by methylmalonyl CoA mutase. It has recently been shown that the *in vitro* proliferation of human and murine leukemia cells is dependent upon both TCII and Cbl (McLean, G. R., Quadros, E. B.,
 5 Rothenberg, S. P., Morgan, A. C., Schrader, J. W., and Ziltener, H. J., 1997 Antibodies to transcobalamin II block *in vitro* proliferation of leukemic cells, *Blood*, 89, 235-242). Several workers have now concentrated on utilizing Cbl conjugates for both radio-imaging and for targeted cancer chemotherapy (Smeltzer, C. C., Pinson, P. R., Munger, J. M., West, F. G., and Grissom, C. B., 1999 Cytotoxicities of two new cobalamin bioconjugates. *Proceedings*
 10 *Ninth International Symposium on Recent Advances in Drug Delivery Systems*, pp 232-3; Canon, M.J., Munger, J. M., West, F. G., and Grissom, C. B., 1999 Synthesis and uptake of radiolabeled cobalamin bioconjugate, *Proceedings Ninth International Symposium on Recent Advances in Drug Delivery Systems*, pp 230-1; Pinson, P. R., Munger, J. M., West, F. G., and Grissom, C. B., 1999 Synthesis of two doxorubicin-cobalamin bioconjugates, *Proceedings*
 15 *Ninth International Symposium on Recent Advances in Drug Delivery Systems*, pp 228-9).

In order for VB₁₂ to co-transport pharmaceuticals across the intestinal epithelial cell layer and into the circulatory system the pharmaceuticals must first be covalently linked to the VB₁₂ molecule. Similarly, in order that VB₁₂ can target an anti-tumour agent to a tumour, the agent must also be
 20 covalently linked to the VB₁₂ molecule. For this to occur, the VB₁₂ molecule itself must first be modified to provide a suitable functional group for conjugation. A carboxylic acid derivative of VB₁₂ is readily achieved by mild acid hydrolysis of the propionimide side chains of the corrin ring structure¹ (see Figure 1). This hydrolysis results in the formation of the "b", "d" and "e" monocarboxylic acids of VB₁₂.² The isolated monocarboxylic acid derivatives can then be
 25 conjugated directly to amino groups of proteins or peptides using commercial carbodiimides such as 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDAC) or dicyclohexylcarbodiimide (DCC) thereby linking the peptide to VB₁₂ via a peptide bond.^{1,3}

A second method of conjugation of peptides to VB₁₂ is by axial substitution of functional
 30 groups onto the Co atom of the corrin ring of the VB₁₂ molecule (see Formula 1). In this method, the axial CN ligand of VB₁₂ can be replaced with a functionalised alkyl chain. This substituted functional group can then be used for conjugation to a peptide or protein using traditional chemical techniques. One major disadvantage of this method, however, is that the

resultant conjugate contains a light sensitive Co-C bond. Thus care must be taken not to expose solutions of the alkylcobalamins to visible light.

Early work by Toraya and Fukui⁴ demonstrated the feasibility of conjugation to VB₁₂ via an ester linkage to the 5'OH of the ribose moiety of the nucleotide ligand. In their work Toraya and Fukui explored the possibility of using this chemistry to form an affinity ligand for purification of diol dehydrase. In order to form the 5'O-ester linkage the authors reacted VB₁₂ with a 54 fold excess of succinic anhydride in a large volume of DMSO (VB₁₂ at 5 mg/ml) plus a large excess of pyridine (128 fold w/w). These authors found that the linkage formed was not only unstable at basic pH, but was also ineffective in purifying the enzyme. Annunziato and co-workers⁵ describe another method of linkage to the 5'OH of the ribose. These workers reacted *p*-maleimidophenyl isocyanate with VB₁₂ and subsequently used the activated VB₁₂ molecule to react with thiolated alkaline phosphatase. Subsequently, Habberfield and co-workers combined the work of Toraya and Fukui⁴ with that of Annunziato *et al.*,⁵ as well as Russell-Jones *et al.*,^{3, 6} and produced conjugates of G-CSF, EPO and consensus interferon to a 5'O-glutaroyl derivative of VB₁₂. The subsequent conjugates were claimed to be active following intraduodenal pump administration to rats of the conjugates pre-complexed to rat IF. In the method described by Habberfield and co-workers, 5 gm of cyanocobalamin (VB₁₂ - 1356 MW) was dissolved in 1,000 ml of DMSO and 200 gm of glutaric anhydride (116 MW) was added in 160 ml of pyridine. The product yield was around 65%. This represents a 468 molar excess of glutaric anhydride to VB₁₂. In the work of Toraya and Fukui,⁴ these workers used 200 mg of cyanocobalamin dissolved in 40 ml DMSO plus 8 grams of succinic anhydride (100 MW) to couple to the hydroxyl group. This represents a 54 fold molar excess of anhydride, with a product yield of 90%. In the method of conjugation described by Russell-Jones and co-workers^{3,6} the VB₁₂ monoacid was prepared by treatment with acid for 72 hrs and subsequent purification on Dowex 1X8 and Dowex 1X2 to afford a yield of only about 5%. In order to link the VB₁₂ monoacid to some peptides and proteins further derivatization of the carboxyl group was often required.

Apart from the methods described by Toraya and Fukui⁴ and Habberfield *et al.*⁷ and Annunziato *et al.*,⁵ there are other methods which could be used to form covalent linkages to the 5'OH group of VB₁₂. These methods are generally used in the preparation of affinity resins by modification of sugar residues resident in agarose. These methods include reaction with oxirane (1,4 butane-diol diglycidyl ether), benzoquinone or cyanuric chloride. These methods have been attempted in the synthesis of VB₁₂ derivatives, however, the yields were either so low as to make the process non-

commercial, or the quantities of reagents employed were so high as to make them similarly non-commercial.

Thus it is an object of the present invention to overcome, or at least alleviate one or more of the
5 abovementioned disadvantages of the prior art. In particular, it is an object of the present invention to provide novel methods for preparing derivatives of VB₁₂ carrier molecules which utilise the 5'OH group of VB₁₂ for chemical bonding with spacer molecules. It is a preferred object of the present invention that the VB₁₂ derivatives are easy to make, obtained in good to high yields and readily purified.

10

SUMMARY OF THE INVENTION

Surprisingly it has been found by the present inventors that VB₁₂ derivatives, which are suitable for conjugation to polymers, nanoparticles and pharmaceutically active agents, are
15 readily prepared by reaction of the 5'OH group on the ribose moiety of VB₁₂ with carbonyl electrophiles.

According to an aspect of the present invention there is provided a method for preparing VB₁₂ derivatives suitable for linking to a polymer, nanoparticle or therapeutic agent, protein
20 or peptide comprising the steps of reacting the 5'OH group of VB₁₂ or an analogue thereof with a bifunctional carbonyl electrophile to form an active intermediate, and subsequently reacting the intermediate with a nucleophilic spacer molecule to yield said VB₁₂ derivative.

According to another aspect of the present invention there is provided a method for preparing
25 a VB₁₂ derivative suitable for linking to a polymer, nanoparticle or therapeutic agent, protein or peptide comprising the steps of reacting a carboxylic acid spacer molecule with a bifunctional carbonyl electrophile to form an active intermediate, and subsequently reacting the 5'OH group of VB₁₂ with the active intermediate to yield said VB₁₂ derivative.

30 There are also provided derivatives of VB₁₂ prepared by the methods of the present invention. These derivatives are ideally linked to a biocompatible polymer or associated with a nanoparticle. These polymers and nanoparticles may be mixed with pharmaceutically acceptable carriers and/or diluents to provide pharmaceutical compositions for therapeutic administration to subjects.

Throughout this specification and the claims which follow, unless the text requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the
5 exclusion of any other integer or step or group of integers or steps.

BRIEF DESCRIPTION OF THE FIGURE

The present invention will now be described with reference to the Figure wherein:

10

Figure 1 is a representation of a VB₁₂ molecule showing three sites for the possible conjugation of agents and peptides to VB₁₂. These sites of conjugation are as follows:

- a) axial conjugation through substitution onto the Co atom of the corrin ring;
- 15 b) direct conjugation following acid modification of the ePropionimide side chain; and
- c) conjugation to the 5'OH group of the ribose moiety of the nucleotide residue.

DETAILED DESCRIPTION OF THE INVENTION

20 The VB₁₂ derivatives of the present invention are suitable for conjugation or linking to polymers, nanoparticles, therapeutic agents, proteins and peptides and other such pharmaceutically active agents. The methods for the production of these VB₁₂ derivatives enable the derivatives to be obtained in generally good to high yields and are of good purity.

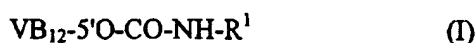
25 In general these derivatives are obtained by dissolving VB₁₂ or an analogue thereof in a solvent, preferably a suitable non-aqueous solvent such as dry DMF, dry THF or dry DMSO, and activating the 5'OH group of VB₁₂ by reaction with a carbonyl electrophile, preferably 1,1'-carbonyldiimidazole at 1-5 molar excess. Quantities above 5 molar excess may be used, however this is generally not required. Preferably VB₁₂ is dissolved at high concentration in
30 DMSO. The activated VB₁₂ intermediate may then be coupled directly to peptides or proteins, or may be reacted with diamino-spacers, or amino-spacer-acids, or alternatively with amino-alkyl chains to form hydrophobic derivatives of VB₁₂ suitable for insertion into the hydrophobic surface of micro- or nanoparticles or into lipids or liposomes.

An alternative method of this invention also utilises the 5'OH group of VB₁₂ in the production of 5'OH ester derivatives of VB₁₂. In the synthesis of the 5'OH ester derivatives an active electrophilic intermediate is first prepared from the reaction of a carboxylic acid spacer molecule with a bifunctional carbonyl electrophile to prepare the active electrophilic
 5 intermediate. VB₁₂ or analogues thereof are then subjected to reaction with the electrophilic intermediate whereby the 5'OH group of VB₁₂ attacks the carbonyl electrophile and displaces a leaving group to yield the VB₁₂ derivative. The VB₁₂ is preferably linked to an amino acid spacer or to an acid lipid in the preparation of the 5'OH ester derivative of VB₁₂. These derivatives have the added advantage that they are easy to make and produce spacers, or
 10 linkages that are readily cleaved by serum esterases to regenerate the native VB₁₂ *in vivo*.

The present inventors have utilised carbonyl electrophiles to enable attack of the weak 5'OH nucleophile by the strongly electropositive carbonyl group in combination with good leaving groups attached to the carbonyl group. The methods overcome problems in the prior art
 15 where strong bases have been used to attach cross-linking agents to the VB₁₂ molecule, these strong bases of which can denature the VB₁₂.

In a preferred embodiment, the carbonyl electrophile is a bifunctional carbonyl electrophile selected from carbonyldiimidazole, phosgene, triphosgene, N,N'-disuccinimidyl carbonate,
 20 carbonyl dipiperidine, 1,1'-carbonyldi(1,2,4-triazole), di(2-pyridyl)ketone, or di(1-benzotriazolyl)carbonate, more preferably carbonyldiimidazole.

The present invention also provides a VB₁₂ derivatives of the formula (I):



25 or a salt thereof, wherein

R¹ is C₁₋₂₄alkyl, C₂₋₂₄alkenyl, C₂₋₂₄alkynyl, C₃₋₈cycloalkyl, (C₃₋₈cycloalkyl)alkyl, amino, -(C₁₋₁₂alkyl)C(O)R², -(C₂₋₁₂alkenyl)C(O)R², -NHC(O)-C₁₋₈alkyl-C(O)NHNH₂ or -CH(R³)C(O)R⁴ all of which optionally may be substituted by one or more groups selected from amino, amido, hydroxy, alkyl, halo, haloalkyl, carboxy, alkoxycarbonyl, acetoxy,
 30 sulfanyl, aryl, arylalkyl and alkylarylalkyl,

R² is amino, hydroxy, C₁₋₆alkoxy or C₂₋₆alkenyloxy,

R³ is an amino acid side chain or a derivative thereof, and

R⁴ is hydroxy, C₁₋₆alkoxy, an amino acid or a peptide.

Preferably R¹ is hexyl, dodecyl, tetradecyl, hexadecyl, octadecyl, aminoethyl, aminobutyl, aminohexyl, aminododecanyl, *t*-butyl-Phe, succinylhydrazidyl, adipylhydrazidyl, Gly-OMe or Gly-OH.

5 The present invention also provides a VB₁₂ derivative of the formula (II):



or a salt thereof, wherein

R¹ is C₁₋₂₄alkyl or C₂₋₂₄alkenyl optionally which may be substituted by one or more groups selected from amino, amido, hydroxy, alkyl, halo, haloalkyl, carboxy, alkoxycarbonyl,

10 acetoxyl, sulfanyl, aryl, arylalkyl and alkylarylalkyl, or

R¹ is -CH(R²)-NHR³,

R² is an amino acid side chain or derivative thereof, and

R³ is hydrogen, an amine protecting group, an amino acid or a peptide.

15 Preferably R¹ is C₈₋₂₄alkyl, C₈₋₂₄alkenyl, or -CH(R²)-NHR³ where R² is glycine and R³ is Boc or hydrogen, or R² is phenylalanine and R³ is Boc or hydrogen. It will be apparent to one skilled in the art that other amino acids or proteins can be used to derivatise the VB₁₂ molecule or analogues thereof. Furthermore, it will be apparent that the amino acids or proteins may require protection of pendant functional groups or other such masking prior to
20 subjecting these reactants to the coupling reactions of the present invention.

The VB₁₂ derivatives of the present invention may be linked to polymers or associated with nanoparticles or the like to prepare vitamin complexes according to standard methods known to those skilled in the art and published in the patent and scientific literature. Examples of
25 such methods may be found in, for example, European Patent No. 0 220 030, Australian Patent No. 664365 and United States Patent Nos. 5449720 and 5548064.

The vitamin complexes are used to deliver agents or active substances, in particular hormones, drugs, prodrugs, enzymes, proteins, peptides, toxins, immunogens or DNA or
30 RNA analogues to subjects. Subjects are preferably vertebrate hosts, more preferably veterinary, domestic and agricultural animals and humans.

The polymers or nanoparticles prepared from the VB₁₂ derivatives of the present invention may be formulated as a pharmaceutical composition by combining the polymers or

nanoparticles with a pharmaceutically acceptable carrier and/or diluent in accordance with standard formulation techniques known to those skilled in the art. The pharmaceutical compositions may be formulated in any acceptable way to meet the desired mode of administration as determined by those skilled in the art.

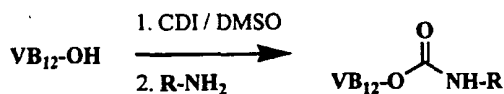
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Major advantages of the methods taught in this specification include the increase in yield of the VB₁₂ derivatives, and cost savings due to the reduction in chemicals used during the activation of the VB₁₂ or the incoming activated acid.

10 The present invention is further described with reference to the following examples which are in no way limiting on the scope of the invention.

Example 1. Preparation of 5'OH-(hexyl)-VB₁₂

15 Materials: VB₁₂ was obtained from Rousell-Uclaf.



VB ₁₂	FW	1355.4
20 CDI	FW	162.2
DMSO		

Solid 1,1'-carbonyldiimidazole (CDI, 260 mg) was added to cyanocobalamin (1.0 g, 0.74 mmol) previously dissolved in dimethylsulfoxide (12 mL) at 30°C and the mixture stirred for 25 min. Hexylamine (2.7 mmol) was added in one portion and stirring continued for a further 24 h at room temperature. The mixture was extracted with phenol / dichloromethane (1:1, 2 × 20 mL) and back extracted with water (2 × 20 mL from 1:4 phenol / dichloromethane). The mixture was purified by phenyl sepharose (50 g) column chromatography, eluting the unmodified VB₁₂ with 25% ethanol and the product with 60% 30 ethanol. The solvent was removed at reduced pressure and the residue was resuspended by sonication for 5 min into acetone (50 mL). The mixture was filtered and the solid washed with acetone and air dried: yield, 60%; mp 213-215°C (dec); MS (ESI) mass calcd for C₇₀H₁₀₁N₁₅O₁₅CoP 1482, found 1505 (M+23)⁺; UV (H₂O) λ₃₆₁ (ε = 10500).

Example 2. Preparation of 5'OH-(d decyl)-VB₁₂

Solid 1,1'-carbonyldiimidazole (CDI, 260 mg) was added to cyanocobalamin (1.0 g, 0.74 mmol) previously dissolved in dimethylsulfoxide (12 mL) at 30°C and the mixture stirred for 25 min. Dodecylamine (2.7 mmol) was added in one portion and stirring continued for a further 24 h at room temperature. The mixture was extracted with phenol / dichloromethane (1:1, 2 × 20 mL) and back extracted with water (2 × 20 mL from 1:4 phenol / dichloromethane). The mixture was purified by phenyl sepharose (50 g) column chromatography, eluting the unmodified VB₁₂ with 25% ethanol and the product with 60% ethanol. The solvent was removed at reduced pressure and the residue was resuspended by sonicated for 5 min into acetone (50 mL). The mixture was filtered and the solid washed with acetone and air dried: yield, 52%; mp 215-218 °C (dec); MS (ESI) mass calcd for C₇₆H₁₁₃N₁₅O₁₅CoP 1566, found 1589 (M+23)⁺; UV (H₂O) λ₃₆₁ (ε = 16900).

15

Example 3. Preparation of 5'OH-(tetradecyl)-VB₁₂

Solid 1,1'-carbonyldiimidazole (CDI, 260 mg) was added to cyanocobalamin (1.0 g, 0.74 mmol) previously dissolved in dimethylsulfoxide (12 mL) at 30 °C and the mixture stirred for 25 min. Tetradecylamine (2.7 mmol) was added in one portion and stirring continued for a further 24 h at room temperature. The mixture was extracted with phenol / dichloromethane (1:1, 2 × 20 mL) and back extracted with water (2 × 20 mL from 1:4 phenol / dichloromethane). The mixture was purified by phenyl sepharose (50 g) column chromatography, eluting the unmodified VB₁₂ with 25% ethanol and the product with 60% ethanol. The solvent was removed at reduced pressure and the residue resuspended by sonication for 5 min into acetone (50 mL). The mixture was filtered and the solid washed with acetone and air dried: yield, 46%; mp 228-233 °C (dec); MS (ESI) mass calcd for C₇₈H₁₁₉N₁₅O₁₅CoP 1595, found 1618 (M+23)⁺; UV (H₂O) λ₃₆₁ (ε = 13000).

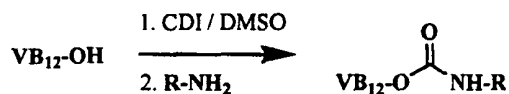
30 Example 4. Preparation of 5'OH-(hexadecyl)-VB₁₂

Solid 1,1'-carbonyldiimidazole (CDI, 260 mg) was added to cyanocobalamin (1.0 g, 0.74 mmol) previously dissolved in dimethylsulfoxide (12 mL) at 30°C and the mixture stirred for 25 min. Hexadecylamine (2.7 mmol) was added in one portion and stirring continued for a

further 24 h at room temperature. The mixture was extracted with phenol / dichloromethane (1:1, 2 × 20 mL) and back extracted with water (2 × 20 mL from 1:4 phenol / dichloromethane). The mixture was purified by phenyl sepharose (50 g) column chromatography, eluting the unmodified VB₁₂ with 25% ethanol and the product with 60% ethanol. The solvent was removed at reduced pressure and the residue was sonicated for 5 min into acetone (50 mL). The mixture was filtered and the solid washed with acetone and air dried: yield, 48%; mp 223-227°C (dec); MS (ESI) mass calcd for C₈₀H₁₂₁N₁₅O₁₅CoP 1623, found 1646 (M+23)⁺; UV (H₂O) λ₃₆₁ (ε = 20000).

10 Example 5. Preparation of 5'-OH-(octadecyl)-VB₁₂

Materials: VB₁₂ was obtained from Roussel-Uclaf.



15

VB₁₂ FW 1355.4

CDI FW 162.2

DMSO

20 VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in dry DMSO (20 ml) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr. The reaction mix was split into 4 equal parts and added to 500 mg of octadecylamine (Aldrich) dissolved in acetone, ethanol, dichloromethane or chloroform. The reaction was allowed to proceed for 2 hours after which the reaction was monitored by 25 TLC and RP-HPLC to determine the quantity of product (5'-OH-(octadecyl)-VB₁₂) which was formed.

The product was then separated from the unreacted VB₁₂ by addition of an equal volume of water and DCM, followed by centrifugation in a Beckman high speed (5K, 10 min). The 30 DCM phase was removed and the product separated from unmodified VB₁₂ by flash column chromatography (isopropanol 50%, ammonia 2%, water 48%) then lyophilised: yield, 66%; mp 220-223°C (dec); MS (ESI) mass calcd for C₈₂H₁₂₅N₁₅O₁₅CoP 1651, found 1674 (M+23)⁺; UV (H₂O) λ₃₆₁ (ε = 17500).

Example 6. Preparation of 5'OH-(aminoethyl)-VB₁₂

VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in dry DMSO (20 ml) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr. Diaminoethane (3.3 equiv) was added to the reaction mix. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue resuspended in acetone (50 mL) by sonication for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by Flash chromatography on a silica column using isopropanol 50%, ammonia 2%, water 48%. The product was then lyophilised: yield, 63%; mp 206-210 °C (dec); TLC (iPrOH 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.22; MS (ESI) mass calcd for C₆₆H₉₄N₁₆O₁₅CoP 1441, found 1441 (M)⁺; UV (H₂O) λ_{361} (ϵ = 19900).

15 Example 7. Preparation of 5'OH-(aminobutyl)-VB₁₂

VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in DMSO (35 mL) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr. Solid diaminobutane (3.3 equiv) was added in one portion. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue in acetone (50 mL) sonicated for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by column chromatography (silica, isopropanol 50%, ammonia 2%, water 48%) then lyophilised: : yield, 70%; mp 242-244 °C (dec); TLC (iPrOH 25 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.08; MS (ESI) mass calcd for C₆₈H₉₈N₁₆O₁₅CoP 1469, found 1469 (M)⁺; UV (H₂O) λ_{361} (ϵ = 15500).

Example 8. Preparation of 5'OH-(t-butyl-Phe)-VB₁₂

30 VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in DMSO (35 mL) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr. Solid t-butyl-Phe (3.3 equiv) was added in one portion. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue in acetone (50 mL) sonicated

for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by Flash column chromatography (silica, isopropanol 50%, ammonia 2%, water 48%) then lyophilysed.

5 Example 9. Preparation of 5'OH-(aminohexyl)-VB₁₂

VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in dry DMSO (20 ml) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr. Diaminohexane (3.3 equiv) was added to the reaction mix as a solid. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue in acetone (50 mL) sonicated for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by column chromatography (isopropanol 50%, ammonia 2%, water 48%) then lyophilysed: yield, 98%; mp 230-233°C (dec); TLC (iPrOH 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.11; MS (ESI) mass calcd for C₇₀H₁₀₂N₁₆O₁₅CoP 1497, found 1497 (M)⁺; UV (H₂O) λ_{361} (ϵ = 17000).

Example 10. Preparation of 5'OH-(aminododecanyl)-VB₁₂

VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in DMSO (35 mL) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 byhr. followed by addition of diaminododecane (3.3 equiv) in one portion. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue resuspended in acetone (50 mL) and sonicated for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by Flash column chromatography (silica resin using isopropanol 50%, ammonia 2%, water 48%) then lyophilysed: yield, 68%; mp 156-158 °C (dec); TLC (iPrOH 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.27; MS (ESI) mass calcd for C₇₆H₁₁₄N₁₆O₁₅CoP 1581, found 1581 (M)⁺; UV (H₂O) λ_{361} (ϵ = 33000).

Example 11. Preparation of 5'OH-(succinylhydrazidyl)-VB₁₂

VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in DMSO (35 mL) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr followed by solid succinyldihydrazide (3.3 equiv) added in one portion. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue in acetone (50 mL) sonicated for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by Flash column chromatography (isopropanol 50%, ammonia 2%, water 48%) then lyophilysed: yield, 68%; mp 206-208 °C (dec); TLC (iPrOH 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.36; MS (ESI) mass calcd for C₆₈H₉₆N₁₈O₁₇CoP 1581, found 1581 (M)⁺; UV (H₂O) λ_{361} (ϵ = 15700).

Example 12. Preparation of 5'OH-(adipylhydrazidyl)-VB₁₂

VB₁₂ (1.0 g, 1.0 equivalent) was dissolved in DMSO (35 mL) at room temperature. Solid carbonyldiimidazole (CDI; 400 mg, 3.3 equivalents) was added and the mixture stirred at room temperature for 1 hr followed by solid adipyldihydrazide (3.3 equiv) added in one portion. The mixture was stirred for 12 h and then poured into acetone / ethyl acetate (1:1, 200 mL) and left to stand. The supernatant was poured off and the residue in acetone (50 mL) sonicated for 5 min. The mixture was filtered onto a sintered glass funnel and the solid washed with acetone. The product was purified by silica column Flash chromatography (isopropanol 50%, ammonia 2%, water 48%) then lyophilysed: yield, 73%; mp 208-210 °C (dec); TLC (iPrOH 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.33; MS (ESI) mass calcd for C₇₀H₁₀₀N₁₈O₁₇CoP 1555, found 1555 (M)⁺; UV (H₂O) λ_{361} (ϵ = 21100).

Example 13. Preparation of ester-linked VB₁₂-phenylalanine

Boc-phenylalanine (1.57 g, 0.0059 mol) and carbonyl diimidazole (1.01 g, 0.0062 mol) were dissolved in DMF (6 ml) and the solution stirred at room temperature for 1 h with vigorous evolution of CO₂. A solution of VB₁₂ (1.0 g) in DMSO (10 ml) was added dropwise to the active ester solution followed by DIEA (1.2 ml, 0.89 g, 0.0069 mol) and stirring was continued at room temperature overnight. Unreacted Boc-Phe, CDI and DIEA were removed by addition of 90 ml acetone to precipitate the VB₁₂. The product was then purified by Flash

chromatography on a silica column (2.5 X 50 cm) using a solvent mixture of 45% butanol, 30% propan-2-ol, 23% DW and 2% NH₄OH. The purified product was lyophilized and the dry powder deprotected by the addition of neat TFA (1 ml /100 mg) for 10 minutes. The product was then precipitated by the addition of ethyl acetate, and dried.

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Example 14 Preparation of ester-linked VB₁₂-glycine

Boc-glycine (1.57 g, 0.0059 mol) and carbonyl diimidazole (1.01 g, 0.0062 mol) were dissolved in DMF (6 ml) and the solution stirred at room temperature for 1 h with vigorous evolution of CO₂. A solution of VB₁₂ (1.0 g) in DMSO (10 ml) was added dropwise to the active ester solution followed by DIEA (1.2 ml, 0.89 g, 0.0069 mol) and stirring was continued at room temperature overnight. Unreacted Boc-Gly, CDI and DIEA were removed by addition of 90 ml acetone to precipitate the VB₁₂. The product was then purified by Flash chromatography on a silica column (2.5 X 50 cm) using a solvent mixture of 45% butanol, 30% propan-2-ol, 23% DW and 2% NH₄OH. The purified product was lyophilized and the dry powder deprotected by the addition of neat TFA (1 ml /100 mg) for 10 minutes. The product was then precipitated by the addition of ethyl acetate, and dried.

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Example 15. Preparation of VB₁₂-glycine acid

Cyanocobalamin (1.0 g, 0.74 mmol) and 1,1'-carbonyldiimidazole (CDI, 260 mg) were added sequentially to dimethylsulfoxide (12 mL) at 30°C and the mixture stirred for 25 min. OMe-Gly (2.7 mmol) was added in one portion followed by triethylamine (200 µL) and the mixture stirred for 24 h at room temperature. The mixture was poured into ethyl acetate (30 mL) and left to stand. The supernatant was poured off and the residue sonicated for 5 min in acetone (50 mL). The mixture was filtered and the solid washed with acetone. The residue was then dissolved in 0.1 M HCl solution and stirred for 30 min. The crude acid was then purified on Dowex 1X4 resin eluting with 2% acetic acid: yield, 95%; mp 239-242 °C (dec); TLC (iPrOH 30/n-BuOH 45/H₂O 25/NH₄OH 2) R_f = 0.41; MS (ESI) mass calcd for C₆₆H₉₀N₁₅O₁₇CoP 1456, found 1456 (M)⁺; UV (H₂O) λ₃₆₁ (ε = 19800).

Example 16. Determination of the relative IF affinity of various 5'O-VB₁₂ derivatives.Reagents

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IF Buffer: BSA (VB₁₂ and IF deficient) BSA (Sigma A-3902) was dissolved at 1 mg/ml in 0.1M potassium phosphate buffer pH 7.5 .

⁵⁷CoVB₁₂: ⁵⁷Co stock (50 µl) (Kodak) was diluted into 50ul of stock in 25ml of IF buffer to give a solution of 1 ng ⁵⁷CoVB₁₂ / 25 ml. 250 ng cold VB₁₂ was added to 25 ml of hot ⁵⁷CoVB₁₂ solution to give a 10 ng/ml solution.

Porcine Intrinsic Factor: Porcine IF (Sigma) was dissolved in IF buffer at 200 Units/ml, and frozen in 500 ul lots (100 IU aliquots) until required.

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BSA-coated charcoal: BSA (1%) was added to an equal volume of 5% charcoal solution of 0.1 M potassium phosphate buffer pH 7.5 and stirred gently for 30 minutes.

Procedure:

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Ten fold up dilutions of VB₁₂ or VB₁₂ derivatives were prepared down to 1 ng/ml in IF buffer. An equal volume of diluted IF was added to each sample and incubated for 20 minutes at room temperature. An equal volume of the BSA-coated charcoal was added to each sample, which was mixed prior to centrifugation. Following centrifugation the supernatant and pellet of each sample were separated and ⁵⁷CoVB₁₂ determined by counting in a gamma counter. Data is represented as the % inhibition of ⁵⁷CoVB₁₂ binding when compared to unmodified VB₁₂.

Compound	% binding relative to vitamin B ₁₂
hexyl-5'O-VB ₁₂	49
dodecyl-5'O-VB ₁₂	35
tetradecyl-5'O-VB ₁₂	4.2
hexadecyl-5'O-VB ₁₂	0.78
octadecyl-5'O-VB ₁₂	0.57
aminoethyl-5'O-VB ₁₂	40
aminobutyl-5'O-VB ₁₂	27
t-butyl-Phe-5'O-VB ₁₂	
aminohexyl-5'O-VB ₁₂	25
aminododecanyl-5'O-VB ₁₂	31
succinylhydrazidyl-5'O-VB ₁₂	37
adipylhydrazidyl-5'O-VB ₁₂	29
phenylalanyl-5'O-VB ₁₂	
glycyl-5'O-VB ₁₂	
HO-Gly-5'O-VB ₁₂	25

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

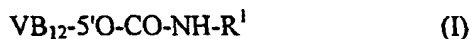
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- 15 5. **Annunziato, M. E., Patel, U. S., Ranade, M., and Palumbo, P. S.** *p*-Maleimidophenyl isocyanate: A novel heterobifunctional linker for hydroxyl to thiol coupling. *Bioconj. Chem.*, 4, 212, 1993.
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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for preparing VB₁₂ derivatives suitable for linking to a polymer, nanoparticle or therapeutic agent, protein or peptide comprising the steps of reacting the 5'OH group of VB₁₂ or an analogue thereof with a bifunctional carbonyl electrophile to form an active intermediate, and subsequently reacting the intermediate with a nucleophilic spacer molecule to yield said VB₁₂ derivative.
2. A method of claim 1, wherein the bifunctional carbonyl electrophile is selected from the group consisting of carbonyldiimidazole, phosgene, triphosgene, N,N'-disuccinimidyl carbonate, carbonyl dipiperidine, 1,1'-carbonyldi(1,2,4-triazole), di(2-pyridyl)ketone or di(1-benzotriazolyl)carbonate.
3. A method of claim 2, wherein the bifunctional carbonyl electrophile is carbonyldiimidazole.
4. A method of claim 1, wherein the nucleophilic spacer molecule is an amino- or hydrazidyl-substituted spacer molecule.
5. A method of claim 4, wherein the spacer molecule is octadecylamine.
6. A method of claim 4, wherein the spacer molecule is diaminoethane.
7. A method of claim 4, wherein the spacer molecule is diaminobutane.
8. A method of claim 4, wherein the spacer molecule is diaminohexane.
9. A method of claim 4, wherein the spacer molecule is diaminododecane.
10. A method of claim 4, wherein the spacer molecule is diaminooctadecane.
11. A method of claim 4, wherein the spacer molecule is an amino acid or a peptide.
12. A method of claim 4, wherein the spacer molecule is a dihydrazide.

13. A method of claim 12, wherein the dihydrazide is succinic acid dihydrazide.
14. A method of claim 12, wherein the dihydrazide is adipic acid dihydrazide.
15. A method for preparing a VB₁₂ derivative suitable for linking to a polymer, nanoparticle or therapeutic agent, protein or peptide comprising the steps of reacting a carboxylic acid spacer molecule with a bifunctional carbonyl electrophile to form an active intermediate, and subsequently reacting the 5'OH group of VB₁₂ with the active intermediate to yield said VB₁₂ derivative.
16. A method of claim 15, wherein the bifunctional carbonyl electrophile is selected from the group consisting of carbonyldiimidazole, phosgene, triphosgene, N,N'-disuccinimidyl carbonate, carbonyl dipiperidine, 1,1'-carbonyldi(1,2,4-triazole), di(2-pyridyl)ketone or di(1-benzotriazolyl)carbonate.
17. A method of claim 16, wherein the bifunctional carbonyl electrophile is carbonyldiimidazole.
18. A method of claim 15, wherein the carboxylic acid spacer molecule is N-Boc-Phe.
19. A method of claim 15 wherein the carboxylic acid spacer molecule is N-Boc-Gly.
20. A VB₁₂ derivative prepared by a method of any preceding claim.
21. A VB₁₂ derivative of the formula (I):



or a salt thereof, wherein

R¹ is C₁₋₂₄alkyl, C₂₋₂₄alkenyl, C₂₋₂₄alkynyl, C₃₋₈cycloalkyl, (C₃₋₈cycloalkyl)alkyl, amino, -(C₁₋₁₂alkyl)C(O)R², -(C₂₋₁₂alkenyl)C(O)R², -NHC(O)-C₁₋₈alkyl-C(O)NHNH₂ or -CH(R³)C(O)R⁴ all of which optionally may be substituted by one or more groups selected from amino, amido, hydroxy, alkyl, halo, haloalkyl, carboxy, alkoxy, carbonyl, acetoxy, sulfanyl, aryl, arylalkyl and alkylarylalkyl, R² is amino, hydroxy, C₁₋₆alkoxy or C₂₋₆alkenyloxy,

R^3 is an amino acid side chain or a derivative thereof, and

R^4 is hydroxy, C_{1-6} alkoxy, an amino acid or a peptide.

22. A VB_{12} derivative of claim 21, wherein R^1 is hexyl, dodecyl, tetradecyl, hexadecyl, octadecyl, aminoethyl, aminobutyl, aminohexyl, aminododecanyl, t-butyl-Phe, succinylhydrazidyl, adipylhydrazidyl, Gly-OMe or Gly-OH.

23. A VB_{12} derivative of the formula (II):



or a salt thereof, wherein

R^1 is C_{1-24} alkyl or C_{2-24} alkenyl optionally which may be substituted by one or more groups selected from amino, amido, hydroxy, alkyl, halo, haloalkyl, carboxy, alkoxy, carbonyl, acetoxy, sulfanyl, aryl, arylalkyl and alkylarylalkyl, or

R^1 is $-CH(R^2)-NHR^3$,

R^2 is an amino acid side chain or derivative thereof, and

R^3 is hydrogen, an amine protecting group, an amino acid or a peptide.

24. A VB_{12} derivative of claim 23, wherein R^1 is C_{8-24} alkyl or C_{8-24} alkenyl.
25. A VB_{12} derivative of claim 23, wherein R^1 is $-CH(R^2)-NHR^3$, R^2 is Gly and R^3 is Boc or hydrogen.
26. A VB_{12} derivative of claim 23, wherein R^1 is $-CH(R^2)-NHR^3$, R^2 is Phe and R^3 is Boc or hydrogen.
27. A polymer or nanoparticle suitable for therapeutic administration to a subject, said polymer or nanoparticle comprising a VB_{12} derivative of any one of claims 20-26.
28. A pharmaceutical composition comprising a polymer or nanoparticle of claim 27 in association with a pharmaceutically acceptable carrier and/or diluent.

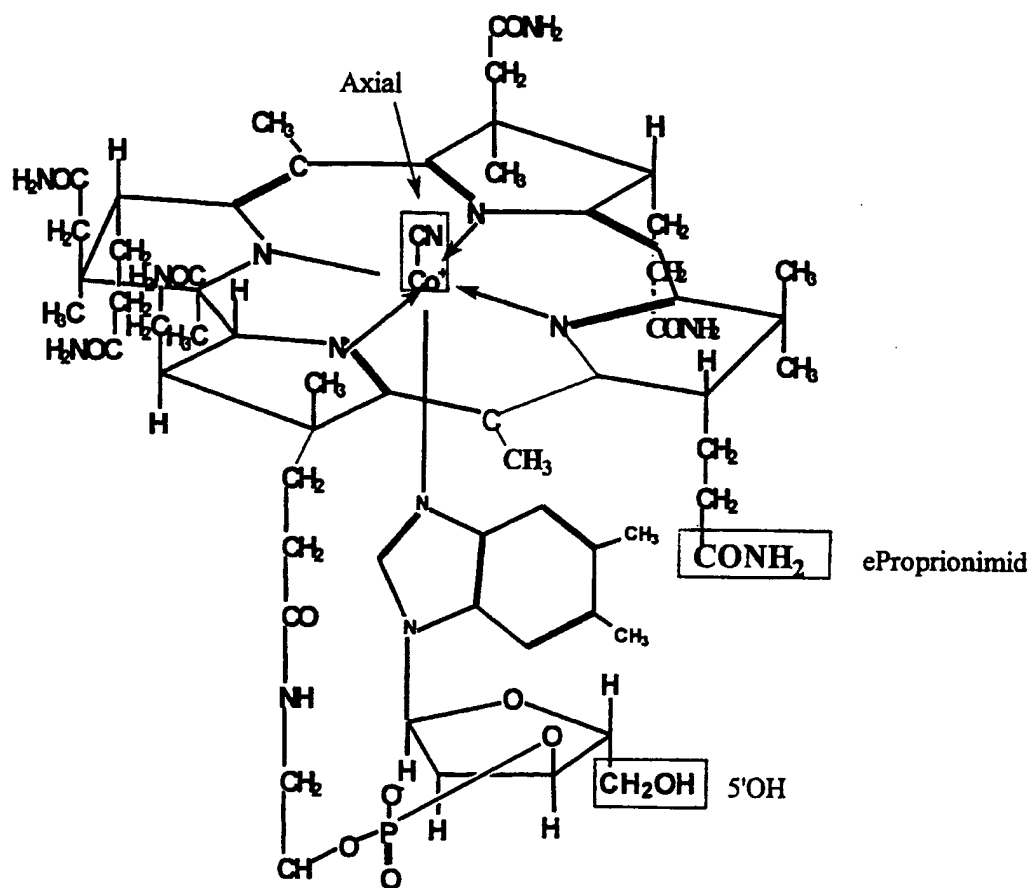



Figure 1. Conjugation sites of VB₁₂

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 99/00462

A. CLASSIFICATION OF SUBJECT MATTER												
Int Cl ⁶ : C07H 23/00, A61K 31/68, 47/48												
According to International Patent Classification (IPC) or to both national classification and IPC												
B. FIELDS SEARCHED												
Minimum documentation searched (classification system followed by classification symbols) IPC C07H 23/00, A61K 31/68, 47/48												
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched												
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN SUBSTRUCTURE SEARCH and MEDLINE: VITAMIN(W) B12, DERIV, PREP												
C. DOCUMENTS CONSIDERED TO BE RELEVANT												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
X	AU A 22835/95 (MORGAN et al) 16 January 1997 Page 13 structure 1, page 14 line 9, pages 33 line 10 to page 36 line 28, Figs 9 to 21	1 to 28										
X	AU B 32015/95 (683581) (AMGEN INC.) 4 March 1996 Page 9, the claims	21 to 28										
X	US A 5510479 (TETSUO TORAYA et al) 23 April 1996 The abstract (X = cyano)	23,24										
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex												
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	"P" document published prior to the international filing date but later than the priority date claimed	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention											
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone											
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art											
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family											
"P" document published prior to the international filing date but later than the priority date claimed												
Date of the actual completion of the international search 27 July 1999		Date of mailing of the international search report -6 AUG 1999										
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer  GAVIN THOMPSON Telephone No.: (02) 6283 2240										

INTERNATIONAL SEARCH REPORT

international application No.

PCT/AU 99/00462

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP A 0 005834 (E.R. SQUIBB & SONS, INC.) 12 December 1979. The abstract, claims	23
X	AU A 76643/96 (RECEPTATGEN CORPORATION) 24 April 1997 Page 12 lines 1 to 8, claim 15	23
X	Clinical Chemistry, Volume 24; No. 2; issued 1978; David B. Endres et al; "A Solid-Phase Radioimmunoassay for Vitamin B12 in Serum, with Use of Radioiodinated Tyrosine Methyl Ester of Vitamin B12"; page 460 to 465 Page 461, left column, line 9	23,25
X	Bioinorganic Chemistry, Volume 4, issued 1975, Tetsuo Toraya et al, "Preparation, Properties and Biological Activities of Succinyl Derivatives of Vitamin B12", pages 245 to 255. Page 246 line 17	23

INTERNATIONAL SEARCH REPORT**Information on patent family members**

International application No.

PCT/AU 99/00462

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
AU	22835/95	CA	2187346	US	5739287	US	5869465
		EP	0754189	US	5840712	WO	95/27723
		JP	10502334	US	5840880		
AU	32015/95 (683581)	CA	2195566	US	5574018	EP	0774976
		JP	9508141	WO	96/04016		
US	5510479	JP	6336494	FR	2705676		
EP	0 005834	AT	295	DE	2960982	US	4209614
		AU	46491/79	IL	57385	ZA	7902064
		CA	1109464	JP	54157600		
AU	76643/96	EP	0856026	WO	97/14740		
END OF ANNEX							